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A TGF-β mediated regulatory mechanism modulates the T cell immune response to rotavirus in adults but not in children

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ABSTRACT

Children with acute RV-gastroenteritis (GE) had low or undetectable levels of circulating IFN-γ^+, IL-13$^+$, IL-2$^+$, IL-10$^+$ or IL-17$^+$ RV-T cells. IFN-γ^+ T cells and low frequencies of IL-10$^+$ and IL-2$^+$ CD4$^+$ T cells were found in adults with RV-GE during acute and convalescence phases, respectively. Circulating single IFN-γ^+>double IFN-γ^+/IL-2$^+$>single IL-2$^+$RV-CD4$^+$T cells were observed in healthy adults. In this group, frequencies of IFN-γ^+ RV-T cells increased after removing CD25$^+$cells, blocking TGF-β with its natural inhibitor, LAP, or inhibiting TGF-βRI signalling pathway with ALK5i. The frequencies of IFN-γ^+ RV-T cells were also incremented in PBMC depleted of CD25$^+$cells and treated with ALK5i, suggesting that TGFβ inhibition may be independent of Treg cells. The ALK5i effect was observed in adults but not in children with RV-GE, who had normal numbers of TGF-β+ Treg cells. Thus, a TGF-β-mediated regulatory mechanism that modulates RV-T cells in adults is not evident in children.

Introduction

Every year, (RV) cause approximately 111 million episodes of gastroenteritis (GE) and 611,000 (range 454,000–705,000) deaths in children younger than 5 years of age worldwide, 1800 of which are estimated to occur in Colombia (Parashar et al., 2006). Advances in our knowledge of the mechanisms that mediate immunity against RV are needed to improve current RV vaccines and/or to develop new RV vaccines (Angel et al., 2007; Franco et al., 2006). Understanding the antiviral T cell response is critical for this purpose, because protective antibodies against RV are T cell dependent and T cells also mediate effector immunity (Angel et al., 2007; Franco et al., 2006).

In the past we have found that both healthy and RV-infected adults have relatively low frequencies of circulating RV-specific CD4$^+$ and CD8$^+$ T (RV-CD4$^+$ and RV-CD8$^+$) cells that secrete interferon gamma (IFN-γ), but not IL-13 or IL-4 (Jaimes et al., 2002; Rojas et al., 2003); also, IL-2$^+$ and IL-10$^+$ CD4$^+$ RV-T cells were variably present and absent, respectively, in a small subset of healthy adult volunteers studied (Narvaez et al., 2005). In children with RV GE, the number of RV-CD4$^+$ T cells that secrete IFN-γ, IL-13 or IL-4 and CD8$^+$ that secrete IFN-γ or IL-4 are even lower or undetectable (Jaimes et al., 2002; Rojas et al., 2003). The low frequencies of RV-T cells could be explained by the following hypothesis: (1) RV triggers specific T cells that secrete cytokines not yet evaluated (Jaimes et al., 2002). (2) In children, the low frequency of RV-T cells could reflect low numbers of T lymphocytes, since absolute lymphopenia (≤2,900/μl) and low percentages of CD4$^+$ and CD8$^+$ T cells, but not B cells, were described in the acute phase in 5/7 of children with RV GE (Wang et al., 2007). (3) RV infection could induce the generation of regulatory T cells (Treg cells). In support of this last hypothesis, it has been shown that Treg cells can suppress T cell responses against RV in mice (Kim et al., 2008), and T cell responses during acute infection with a non persistent virus like dengue in humans (Luhn et al., 2007). For RV, this hypothesis gains particular relevance since intestinal dendritic cells (most probably the first antigen presenting cell [APC] to handle the virus) from mice (Coombes et al., 2007; Sun et al., 2007) and humans (Rimoldi et al., 2005) are prone to induce anti-inflammatory immune responses and in mice promote *de novo* generation of FoxP3$^+$ Treg cells through mechanisms involving Transforming Growth Factor-beta (TGF-β) and retinoic acid (Coombes et al., 2007; Sun et al., 2007).

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Here we tested these three hypotheses by determining: (1) The frequencies of circulating RV-CD4$^+$ and CD8$^+$ T cells that secrete IFN-γ, IL-13, IL-2, IL-10 or IL-17 in children and adults with or without RV GE. (2) The relative and absolute numbers of circulating leukocytes, lymphocytes and Treg cells from children with acute RV GE. (3) The frequencies of circulating IFN-γ$^+$ RV-CD4$^+$ and CD8$^+$ T cells after removal of Treg cells (CD25$^+$ cells) or inhibition of their TGF-β dependent function by two approaches: blocking TGF-β with its natural inhibitor LAP, or inhibiting the signalling pathway of the TGF-β receptor TGF-βRI/ALK5 with the synthetic inhibitor SB431542 (ALK5i).

Results

Frequencies of RV-T cells in healthy adults and adults with RV GE

Of the cytokine producing T cells evaluated in healthy adults volunteers, only RV-specific IFN-γ$^+$ and IL-2$^+$ CD4$^+$ T cells were detected in 14/21 (66.7%) and 6/10 (60%) individuals, respectively, at frequencies ≤0.65% (Fig. 1A). IFN-γ$^+$ and IL-2$^+$ RV-CD8$^+$ T cells were observed in 8/20 (40%) and 1/9 healthy adult volunteers, respectively (Fig. 1B). The analysis of multifunctional RV-specific T cells (producing simultaneously more than one cytokine), in a subset of healthy adult volunteers from Fig. 1, showed that the majority of RV-CD4$^+$ T cells produce only IFN-γ and that most of the IL-2$^+$ RV-CD4$^+$ cells were simultaneously secreting IFN-γ (Fig. 2A). In contrast, for SEB-stimulated PBMC, the CD4$^+$ T cells were mostly single IL-2$^+$ followed by the IFN-γ$^+$ and IL-2$^+$ double positive cells (IFN-γ$^+$/IL-2$^+$) and single IFN-γ$^+$ cells (Fig. 2C). For both, RV- and SEB-CD8$^+$ T cells, single IFN-γ$^+$ were the predominant population, followed by single IL-2$^+$ and IFN-γ$^+$/IL-2 cells (Figs. 2B, D). Thus, compared to SEB stimulated cells, the profile of IFN-γ$^+$ and IL-2$^+$ RV-CD4$^+$ is different and that of CD8$^+$ is similar. RV specific CD4$^+$ and CD8$^+$ IL-10$^+$ (Figs. 1A–B), IL-13$^+$ or IL-17$^+$ (data not shown) were not detected in 6–7 healthy adult volunteers.

In 4/4 adults with acute RV GE, IFN-γ$^+$ and IL-10$^+$ RV-CD4$^+$ T cells were found at higher percentages ($P=0.016$ and $P=0.009$, respectively; Mann–Whitney tests) than those detected in healthy volunteers and IL-2$^+$ were not detected in 3/3 patients (Fig. 1A). During the convalescence phase, IFN-γ$^+$ RV-CD4$^+$ T cells were detected in 5/5, IL-10$^+$ were undetectable in the four patients studied and IL-2$^+$ CD4$^+$ T cells were present in 3/5 patients (Fig. 1A). RV-CD8$^+$ IFN-γ$^+$, IL-2$^+$ and IL-10$^+$ T cells were seen in 2/3, 0/3 and 1/3 patients with acute RV GE (Fig. 1B) respectively, and during the convalescence phase only 2/3 secreted IFN-γ$^+$ (Fig. 1B). Cells stimulated with SEB were used as a positive control in all experiments, and the expected frequencies of cytokines producing CD4$^+$ and CD8$^+$ T cells (Table S2) were found (Jaimes et al., 2002).

Groups of children studied

Three groups of children with GE were studied: group A, children without evidence of previous RV infection (plasma RV-IgA$^-$) and without RV GE ($n=15$); group B, children with evidence of previous RV infection (plasma RV-IgA$^+$) but without RV GE ($n=21$), and group C children with RV GE ($n=17$). Demographic and clinical data of these children are summarized in supplemental material Table S1. Boys were more frequent than girls in groups A and C ($P=0.010$ and 0.016 respectively; chi square test). The days after onset of diarrhea (DAOD) were lower in children from group C compared to children from groups A and B ($P=0.010$; Kruskall–Wallis test; $P=0.018$ and $P=0.004$, respectively; Mann–Whitney tests). No differences were observed in age ($P=0.263$; Kruskall–Wallis test), the Ruuska–Vesikari score ($P=0.844$; Kruskall–Wallis test) or breast feeding among the three groups ($P>0.05$; chi square test). When children without RV GE from A and B groups were combined (group A + B), and compared to group C, children with RV GE, the latter had lower DAOD ($P=0.024$; Mann–Whitney test). Differences in gender, the Ruuska–Vesikari score and breast feeding between groups A + B and C were not significant.

Circulating IL-2$^+$, IL-10$^+$ or IL-17$^+$ RV-CD4$^+$ and CD8$^+$ T cells are low or absent in peripheral blood from children with acute GE

In children with acute GE, from groups A, B and C, the frequencies of circulating IFN-γ$^+$, IL-13$^+$, IL-2$^+$, IL-10$^+$ and IL-17$^+$RV-CD4$^+$ T cells were low (<0.06) in few children or absent in the majority, and no statistical differences were seen between the three groups (Fig. 3). Only 2/12 children from each group B and C had IFN-γ$^+$RV-CD4$^+$ T cells (Fig. 3). RV-CD8$^+$ T cells were studied in a subset of children of

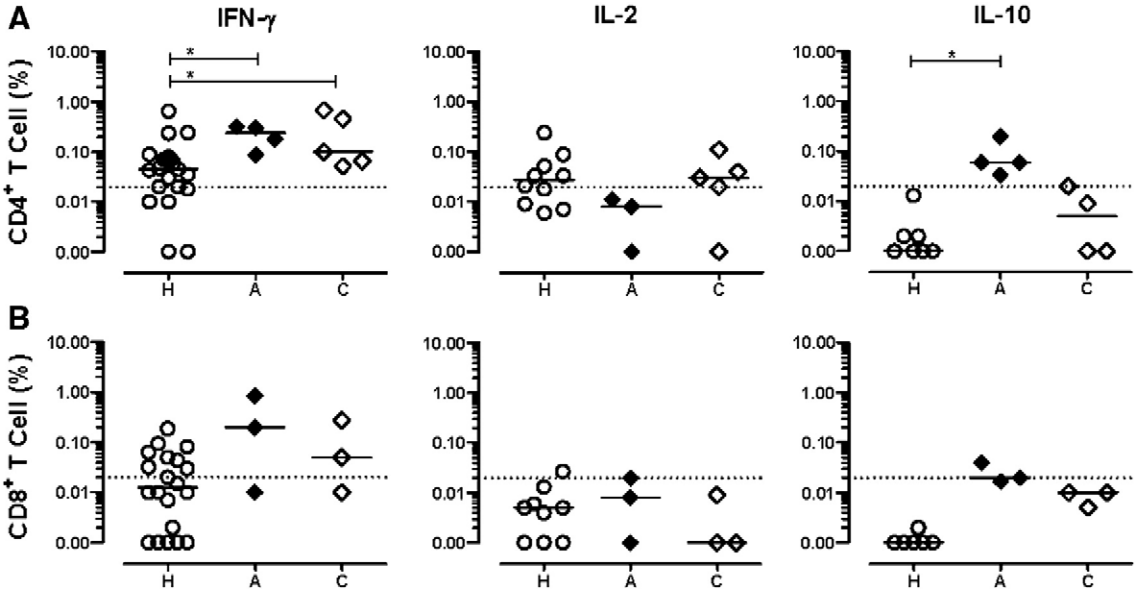

Fig. 1. Frequencies of RV-specific CD4$^+$ and CD8$^+$ T cells in healthy adult and adults with acute RV GE. PBMCs from healthy adults (H) and adults with RV GE during acute (A) or convalescence phase (C) were cultured with RRV (MOI:7) or mock in the presence of anti-CD28 and anti-CD49d MAbs for 10 h; brefeldin A was added during the last 5 h. IFN-γ, IL-2 and IL-10 producing T cells were evaluated by ICS and flow cytometry. (A) CD4$^+$ and (B) CD8$^+$ RV-specific T cells are shown as net percentages (RRV minus mock). The dotted line represents the limit detection. *$P<0.05$; Mann–Whitney test.

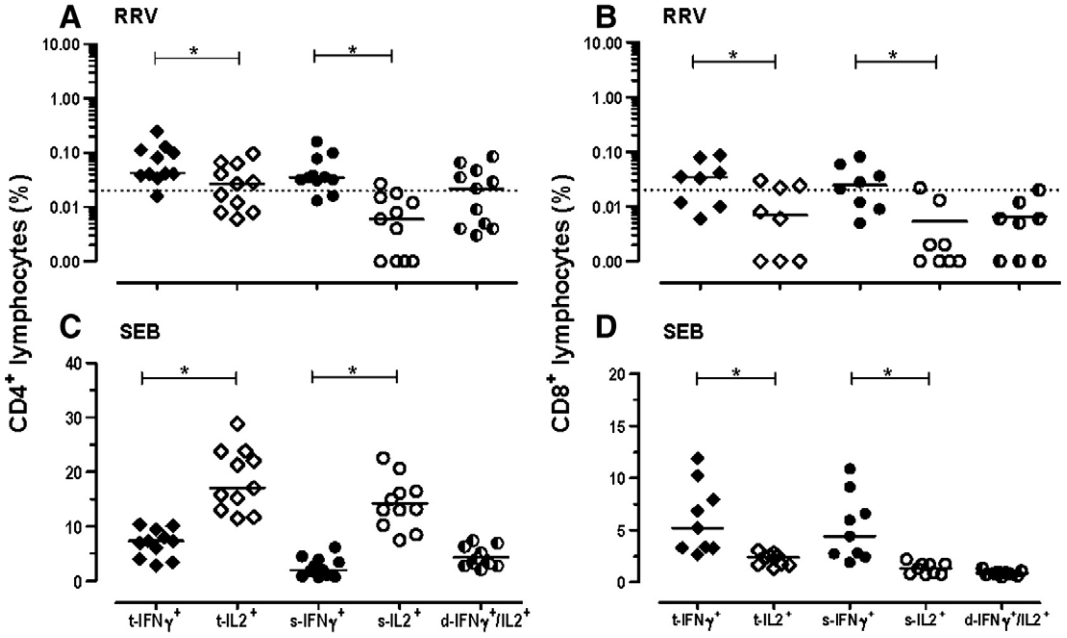

Fig. 2. Frequencies of RV and SEB-specific multifunctional IFN-γ^+/IL-2^+ $CD4^+$ and $CD8^+$ T cells in healthy adult volunteers. PBMC were cultured with RRV (MOI:7), mock or SEB, in the presence of anti-CD28 and anti-CD49d MAbs for 10 h; brefeldin A was added during the last 5 h. IFN-γ and IL-2 were evaluated in $CD4^+$ and $CD8^+$ cells by ICS and flow cytometry. Using boolean gates, the frequencies of $CD4^+$ and $CD8^+$ T cells secreting total IFN-γ (t-IFNγ^+), total IL-2 (t-IL2^+), only IFN-γ^+ (s-IFNγ^+), only IL-2^+ (s-IL2^+) or both cytokines (d-IFNγ^+/IL2^+) were evaluated. (A–B) RV-$CD4^+$ and $CD8^+$ T cells: Net percentages (RRV minus mock) values are shown. (C–D) SEB-stimulated $CD4^+$ and $CD8^+$ T cells. The dotted line represents the limit detection. * $P<0.05$; Wilcoxon test.

the three groups and only 1/4 children from group B had IFN-γ^+ RV-$CD8^+$ T cells (data not shown). These findings suggest that few children with GE and past (group B) or present (group C) RV infection have circulating RV-T cells. Expected frequencies of IFN-γ^+, IL-13^+, IL-2^+, IL-10^+ or IL-17^+ $CD4^+$ and $CD8^+$ T cells were observed in response to the polyclonal stimulus with SEB (Jaimes et al., 2002), with similar values in the three study groups (Table S3).

T cell lymphopenia is observed in a subset of children with acute RV GE

Lymphopenia has been described in a subset of children with acute RV GE (Wang et al., 2007). To determine if lymphopenia (<2900 lymphocytes/μl) could be related to the low numbers of RV-T cells, we determined absolute lymphocyte counts in children. We found absolute lymphopenia in 5/12 (41.6%) children with RV acute GE (Group C) and only in 1/25 from group A + B ($P<0.01$; Fisher exact test) (Fig. 4). The frequencies of $CD3^+$, $CD4^+$ and $CD8^+$ were comparable between groups A + B and C (Fig. 4). No differences were observed for numbers of leukocytes, neutrophils or monocytes between groups A + B vs. C (Fig. 4).

Depletion of $CD25^+$ cells from PBMC of healthy adult increases the frequency of RV-T cells

To explore the role of $CD25^+$ Treg cells on the RV-T cell response, the frequency of RV-specific IFN-γ^+ T cells was evaluated in $CD25^+$-

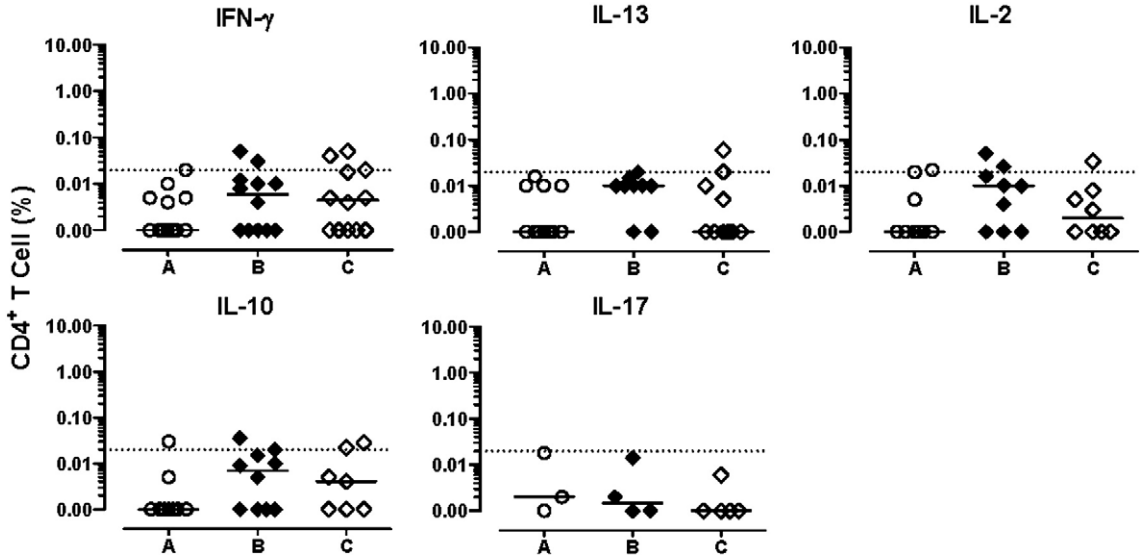

Fig. 3. Frequencies of RV-$CD4^+$ T cells secreting cytokines in children with acute GE. PBMC from children from groups A, B and C, were cultured with RRV (MOI:7) or mock in the presence of anti-CD28 and anti-CD49d MAbs for 10 h; brefeldin A was added during the last 5 h. Frequencies of IFN-γ, IL-13, IL-12, IL-10 and IL-17 producing $CD4^+$ T cells were evaluated by ICS and flow cytometry. Net percentages (RRV minus mock) values are shown. The dotted line represents the limit detection.

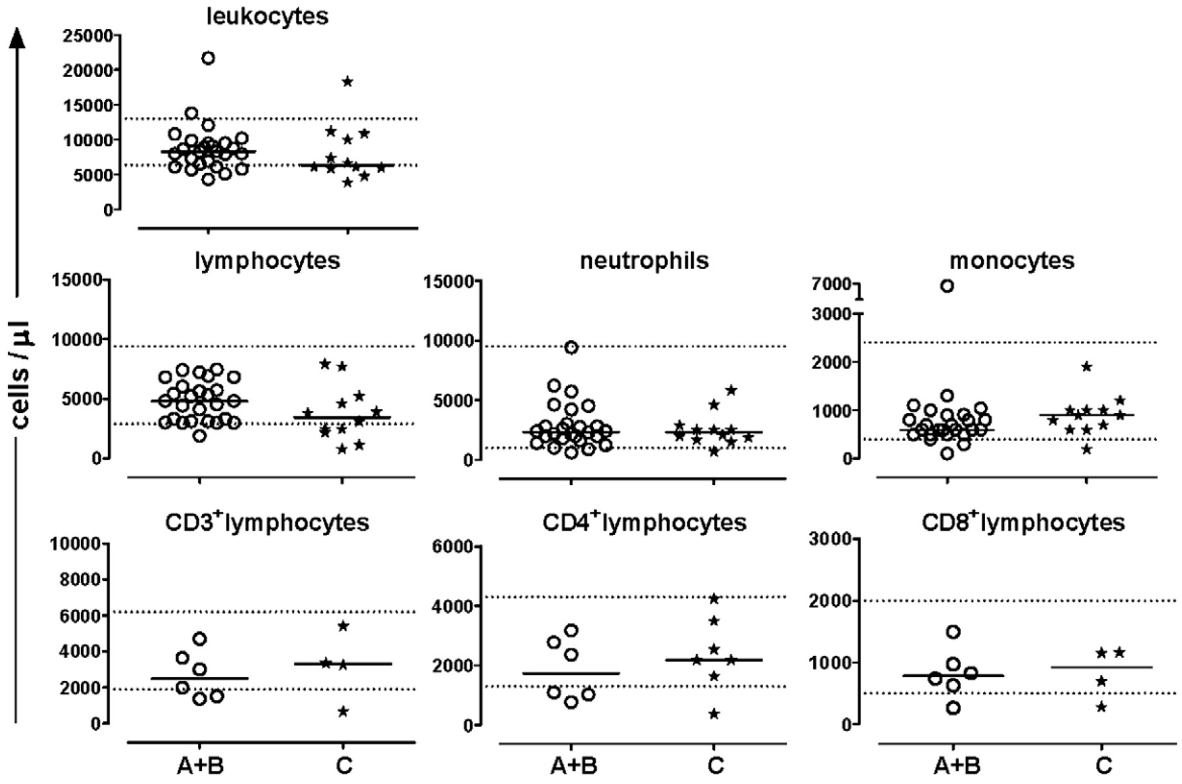

Fig. 4. Absolute counts for peripheral blood leukocytes subsets in children with acute GE. Absolute counts for total leukocytes, lymphocytes, neutrophils and monocytes from children from groups A, B and C, were obtained from electronic analyzers. Frequencies of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were determined by flow cytometry. Absolute values for $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ subsets were estimated from absolute lymphocyte counts. The dotted lines limit the region of reference values for children under 2 years (Shearer et al., 2003).

depleted and not depleted PBMC from healthy adult volunteers. The frequency of IFN-γ^+ RV-$CD4^+$ lymphocytes was significantly ($P=0.0053$, Wilcoxon test; $n=13$) higher after removing $CD25^+$ cells (Fig. 5A). For RV-$CD8^+$ lymphocytes, the results were not significant but 4/13 volunteers showed increments in the percentage of IFN-γ^+ RV-$CD8^+$ cells after $CD25^+$ cell depletion (Fig. 5B). A comparable (not statistically significant) trend was observed for $CD4^+$ and $CD8^+$ T cells in similar experiments using human Wa strain RV ($n=5$; data not shown). Cultures with polyclonal SEB stimulus showed small but not significant increments in percentages of IFN-γ^+ $CD4^+$ and $CD8^+$ lymphocytes in 8/13 healthy volunteers (Fig. S1A–B). Thus, in healthy adult volunteers, the IFN-γ^+ memory T cell response against RV is modulated by $CD25^+$ cells.

TGF-β modulates RV-T cells in healthy adults

One of the mechanisms that $CD25^+$ and $CD25^-$ Treg cells use for their regulatory function involves membrane bound TGF-β (Nakamura et al., 2004). To determine if TGF-β modulates the RV T cell response, T cells were stimulated with RV in the presence or absence of its natural inhibitor rhLAP, which can reverse the suppression exerted by TGF-β dependent Treg cells (Nakamura et al., 2004). Significantly higher percentages of IFN-γ^+ RV-$CD4^+$ and $CD8^+$ lymphocytes were observed in the presence of rhLAP (Fig. 5C–D). The treatment with rhLAP also increased the frequencies of IFN-γ^+ T cells in PBMC cultures stimulated with SEB (Fig. S1C–D). In similar cultures ($n=3$) stimulated with the human Wa RV strain, rhLAP also increased the frequencies of IFN-γ^+ $CD4^+$ and $CD8^+$ T cells (data not shown). To further clarify the role of TGF-β in modulating RV-T cells, PBMC were preincubated with and without SB431542 (ALK5i, a synthetic inhibitor of the intracellular signalling of the TGF-β receptor) (Oida et al., 2006) before stimulation with RV. Significant increments in the frequencies of IFN-γ^+ RV $CD4^+$ ($P=0.005$, Wilcoxon test; $n=11$) and in IFN-γ^+ RV $CD8^+$ ($P=0.008$, Wilcoxon test; $n=11$) T cells were observed in cultures of PBMC treated with the ALK5i (Fig. 5E–F). A similar trend was observed in response to SEB (Fig. S1E–F). To determine if the TGF-β was derived from $CD25^+$ Treg cells (Laouar et al., 2005), the frequencies of IFN-γ^+ RV-T cells were evaluated after simultaneous $CD25^+$ cell depletion and treatment with ALK5i. Percentages of IFN-γ^+ $CD4^+$ and $CD8^+$ were increased in cultures both depleted of $CD25^+$ cells and treated with ALK5i, compared to only $CD25^+$ depleted and only ALK5i treated cells, in most (5/8) volunteers (Fig. 5G–H). Variable results were observed with SEB stimulated cells (Fig. S1G–H). Thus, the IFN-γ^+ RV-memory T cell response in healthy adults may be regulated by $CD25^+$ Treg cells by a TGF-β independent mechanism, and also by TGF-β, independently of $CD25^+$ Treg cells.

TGF-β modulates the RV-T cell response in adults but not in children with acute RV GE

To evaluate if the TGF-β-dependent regulatory mechanism modulates RV-T cells of adults and children with RV GE, their RV-T cells were studied in the presence or not of ALK5i. In adults in acute and convalescence phases of RV GE, the frequencies of IFN-γ^+ RV-$CD4^+$ were higher in the presence of ALK5i in 4 volunteers studied (Fig. 6A). A similar trend was observed for IFN-γ^+ RV-$CD8^+$ T cells in 3 individuals (Fig. 6B). Increments were also observed in response to SEB for both $CD4^+$ and $CD8^+$ lymphocytes during the acute and convalescence phases (Fig. S2A–B). In children from groups A, B and C, no increments in the frequencies of IFN-γ^+ RV-$CD4^+$ T cells were observed in the presence of ALK5i (Fig. 6C). A significant increment was observed in the frequency of IFN-γ^+ $CD4^+$ lymphocytes in response to SEB in children from group A and A + B ($P=0.016$ and $P=0.012$, respectively; Wilcoxon tests) but not in children from groups B and C (Fig. S2C). The frequencies of IL-2^+, IL-13^+ or IL-10^+ RV-$CD4^+$ T cells were not increased after ALK5i treatment of cells from

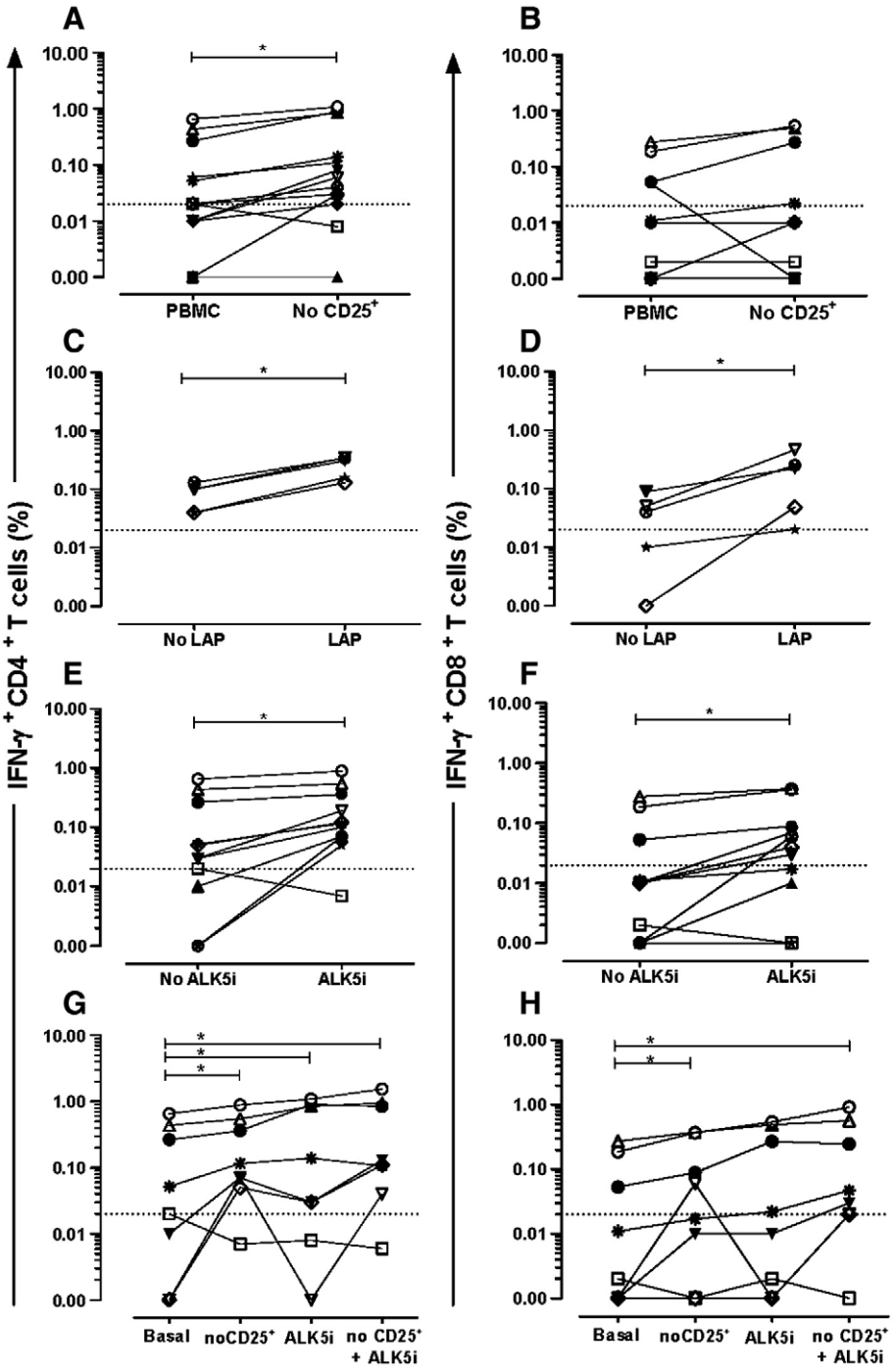

Fig. 5. CD25$^+$ Treg cells and a TGFβ-mediated mechanism modulate RV-T responses in healthy adults. Total PBMC or PBMC with different treatments (see below) were stimulated with RRV (MOI:7) or mock in the presence of anti-CD28 and anti-CD49d MAbs for 10 h; brefeldin A was added during the last 5 h. CD4$^+$ and CD8$^+$ IFN-γ$^+$ T cells were quantified using ICS and flow cytometry. Net percentages (RRV minus mock) of IFN-γ$^+$ lymphocytes are shown. (A, B) PBMC without CD25$^+$ cells. (C, D) PBMC exposed to LAP for 10 min prior to stimulation. (E–F) PBMC exposed to ALK5i for 30 min prior to stimulation. (G, H) PBMC exposed to ALK5i or CD25$^+$ cells depletion or both treatments simultaneously prior to stimulation. (A, C, E and G) Percentages of IFN-γ$^+$ CD4$^+$ T cells. (B, D, F and H) Percentages of IFN-γ$^+$CD8$^+$ T cells. The dotted line represents the limit detection. *$P<0.05$; Wilcoxon test.

GE-affected adults or children (data not shown). These results suggest that the TGF-β dependent regulatory mechanism that modulates IFN-γ$^+$ RV-T cells becomes evident with age, being apparent in adults but not children with present (group C) or past (group B) RV infections.

Numbers of circulating CD4$^+$, CD25$^+$, LAP$^+$ (TGF-β$^+$) Treg cells are comparable in children with RV or non-RV GE

To determine if the absence of the regulatory-TGF-β dependent mechanism in children, described above, was related to a diminished frequency of their circulating Treg cells, the relative and absolute frequencies of circulating CD4$^+$CD25$^+$ (the most frequently evaluated subset of Treg cells) (Sakaguchi et al., 2006), CD4$^+$CD25$^+$CD127^{low} (a subset equivalent to classical FoxP3$^+$ Treg cells) (Seddiki et al., 2006) and CD4$^+$CD25$^+$CD127^{low}LAP$^+$ (Treg cells that express surface TGF-β) (Ito et al., 2008) T cells were quantified (Fig. 7 and S3). All Treg cells subsets were observed in peripheral blood from children with acute RV GE with relative and absolute counts similar to those from children with non-RV acute GE (Fig. 7). CD45RA was expressed in most of the CD4$^+$CD25$^+$CD127^{low} Treg cells and in approximately 40%

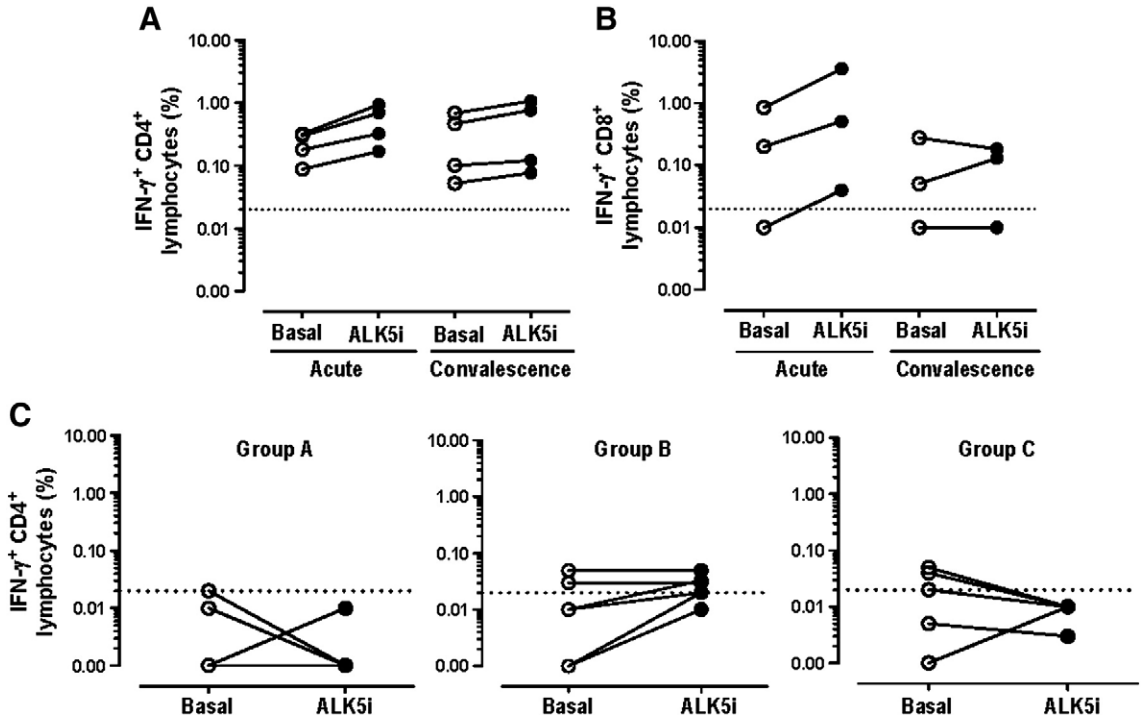

Fig. 6. ALK5i increases the frequencies of RV- IFN-γ^+ $CD4^+$ and $CD8^+$ lymphocytes in adults but not in children with RV GE. (A, B) PBMC drawn from adults with acute RV GE during acute and convalescence phases; and (C) PBMC from children from groups A and B and C were exposed or not to ALK5i for 30 min prior to adding RRV (MOI = 7) or mock in the presence of anti-CD28 and anti-CD49d MAbs for 10 h. Brefeldin A was added during the last 5 h. IFN-γ^+ lymphocytes were evaluated using ICS and flow cytometry. Net percentages (RRV minus mock) of IFN-γ^+ $CD4^+$ lymphocytes (A) and $CD8^+$ lymphocytes (B) from adults and IFN-γ^+ $CD4^+$ lymphocytes from children (C) are shown. The dotted line represents the limit detection.

of $CD4^+CD25^+CD127^{low}LAP^+$ (Fig. S3). Thus, although TGF-β^+ $CD25^+$ Treg cells are present in the peripheral blood from children with RV GE with frequencies similar to those detected in children with non-RV GE, this cytokine is not responsible for the low frequency of IFN-γ^+ RV-$CD4^+$ T cells detected in short term cultures of PBMC from children with acute RV GE.

Discussion

We have extended our previous studies to show that, like for IFN-γ, IL-13 and IL-4 (Jaimes et al., 2002; Rojas et al., 2003), very few or no IL-2^+, IL-10^+, IL-17^+ RV-$CD4^+$ and $CD8^+$ T cells circulate in children with acute RV GE (Fig. 3 and data not shown). In healthy adults, most RV-$CD4^+$ cells secrete INF-γ, few secrete simultaneously IFN-γ and IL-2 and none IL-2 alone (Fig. 2A). In adults with acute RV GE, low levels of IL-10^+ and IL-2^+ RV-$CD4^+$ cells were found during acute and convalescence phases, respectively (Fig. 1A). In adults, the IFN-γ^+ RV-$CD4^+$ and in some cases $CD8^+$ T cell response is partially regulated by $CD25^+$ T cells (Fig. 5A–B) and independently by a TGF-β-dependent mechanism (Fig. 5G–H); such mechanism is not observed in children with past or present RV infection (Fig. 6C).

As previously noted (Jaimes et al., 2002; Rojas et al., 2003), the frequency of IFN-γ^+ RV-$CD4^+$ and -$CD8^+$ T cells in acutely infected adults (Fig. 1A–B) is much lower than in patients with acute EBV (Amyes et al., 2003; Hoshino et al., 1999), and CMV (Harari et al., 2005; Lidehall et al., 2005) which are accompanied by important viremia, and where virus specific $CD8^+$ T cells can be as high as 60% of blood T cells. Unlike for children (Table S1), we have not detected antigenemia in RV infected adults (data not shown). Thus, the frequencies of IFN-γ^+ RV-$CD4^+$ and $CD8^+$ T cells in adults with acute RV GE, may be lower because important systemic dissemination of virus does not take place. The frequencies of RV IFN-γ^+-$CD4^+$ and -$CD8^+$ T cells circulating in healthy adults (Fig. 1A–B) although present only in a subset of volunteers (66,7% and 40% for $CD4^+$ and $CD8^+$ T cells, respectively) in general are comparable to those specific for some non mucosal viruses (Harari et al., 2005; Nanan et al., 2000), and to responses to several mucosal respiratory viruses (de Bree et al., 2007; Heidema et al., 2008; Kannanganat et al., 2007; Tilton et al., 2007).

IL-2 has been previously shown in supernatants of PBMC stimulated with RV from healthy adults but not cord blood (Mesa et al., 2007; Yasukawa, Nakagomi, and Kobayashi, 1990). Also, IL-2 is produced by $CD4^+$ T cells stimulated with RV-exposed mDC in 2 of 4 healthy volunteers studied (Narvaez et al., 2005). Here we showed that RV-$CD4^+$ T cells from healthy adults predominantly secrete IFN-γ, very few secrete both IFN-γ and IL-2, (the latter only in 55% of volunteers), and cells secreting only IL-2 are not detectable (Fig. 2A). RV IL-2^+ $CD4^+$ T cells were evidenced during the convalescence phase in 3/5 adults with RV GE but not in 3/3 adults studied during the acute phase (Fig. 1A). Contrary to RV but similar to SEB (Fig. 2A, C), IL-2^+ or IFN-γ^+IL-2^+ double producers $CD4^+$ T cells are more frequent than IFN-γ^+ single producer in response to viruses that are efficiently cleared (vaccinia and influenza) (Harari et al., 2005; Kannanganat et al., 2007; Tilton et al., 2007). The same is true, but to a lower degree, with viruses that generate persistent infections but relatively low levels of circulating antigen (CMV, EBV) (Harari et al., 2005; Kannanganat et al., 2007; Tilton et al., 2007). The presence of a dominant single IFN-γ^+ $CD4^+$ T cell response, like we observe for RV (Fig. 2A), has been associated with chronic infections with persistent antigen (HIV, HCV) and in response to non protective vaccines (Harari et al., 2005; Seder et al., 2008). Thus, the lack of circulating IFN-γ^+/IL-2^+ RV-$CD4^+$ T cells in approximately 40% of healthy adults (Fig. 2A) and 3/3 adults with RV GE (Fig. 1A) could be related to a higher susceptibility to RV infection. In the convalescence phase, the IL-2^+ RV-$CD4^+$ subset was detected in 3/5 subjects examined, suggesting that these cells appear upon multiple infections.

RV-$CD4^+$ T cells from adults with RV GE secrete IL-10 during acute phase, but not during the convalescence phase after infection

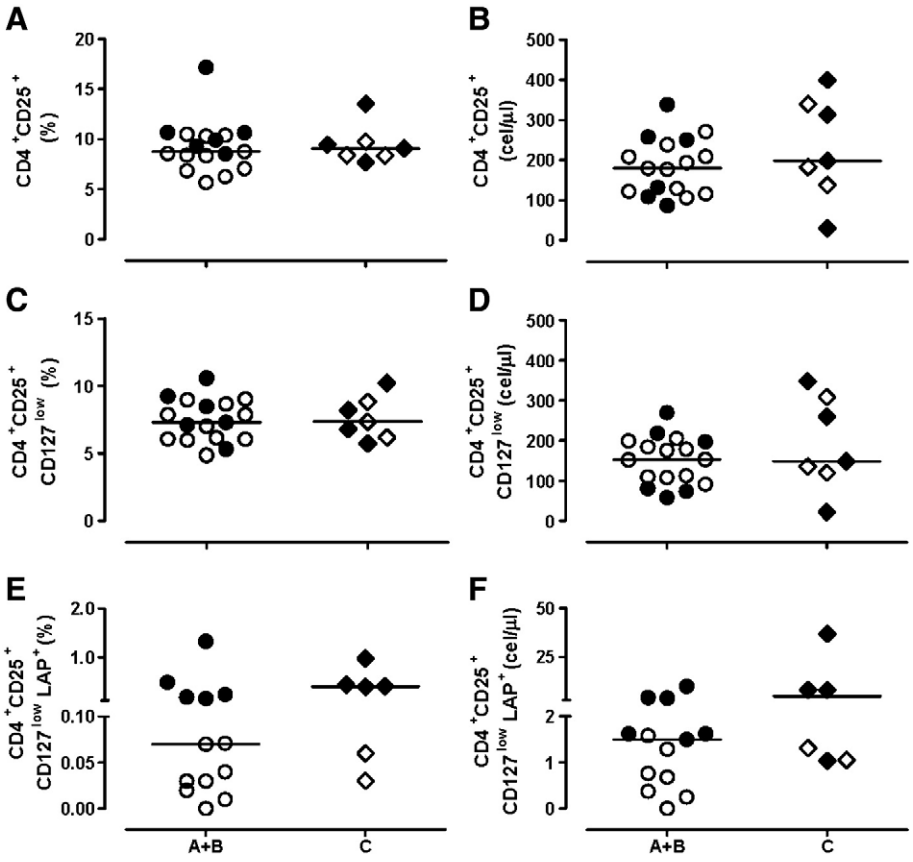

Fig. 7. Relative and absolute counts of Treg cells subsets in children with acute GE. Relative (A, C, E) and absolute (B, D, F) numbers of Treg cells subpopulations were determined in PBMC from children with acute GE from groups A + B (circles) and C (diamonds). The cells were stained following protocol 1 (open symbols) or protocol 2 (filled symbols), described in Materials and methods and analyzed as described in Fig. S3A or S3B. Stain 1 and Stain 2 include polyclonal and MAbs against LAP, respectively (described in Materials and methods). Subpopulations of Treg cells were defined as (A, B) $CD4^+CD25^+$, (C, D) $CD4^+CD25^+CD127^{low}$ and (E, F) $CD4^+CD25^+$ $CD127^{low}LAP^+$. Absolute counts were estimated based on lymphocyte numbers from Fig. 4. Similar results to those shown for $CD4^+CD25^+$ cells (upper row) were obtained upon analysis of $CD4^+$ $CD25^{hi}$ cells (data not shown). Bars represent median values.

(Fig. 1A), suggesting a transient regulatory mechanism. Among $CD4^+$ T cells, IL-10 is produced by Th1 (Del Prete et al., 1993; Jankovic et al., 2007; O'Garra and Vieira, 2007) and Th2 (Street and Mosmann, 1991) effector cells, and natural (Asseman et al., 1999; Ito et al., 2008) and adaptive Tr1 Treg cells (Groux et al., 1997; Roncarolo et al., 2006). To establish, whether Th1, Tr1 or other subsets of RV $CD4^+$ T cells are the IL-10 producing cells in adults with acute RV GE (Fig. 1A) will require additional studies.

In contrast to the findings in adults, only very few children with RV GE had low levels of IFN-γ^+, IL-2^+ or IL-10^+ RV-$CD4^+$ T cells and none had IL-17^+ cells (Fig. 3A). Having broadened our panel of cytokines to evaluate RV-T cells, it seems unlikely that, as previously proposed (Jaimes et al., 2002), the low frequencies of RV-T cells observed in children are due to the fact that they secrete other cytokines than those analyzed. Moreover, a general dysfunction of T cells during RV GE also does not seem a complete explanation for this phenomenon, since SEB responses in children with RV GE were comparable to children without RV GE (Table S3). In addition, although we found absolute lymphopenia in 5/12 (41.6%) children with acute RV GE (Fig. 4), a somewhat lower value than that previously reported 5/7 (Wang et al., 2007), low frequencies of RV IFN-γ^+ $CD4^+$ T cells were equally observed in children with low or normal absolute counts of lymphocytes (data not shown).

The removal of $CD25^+$ cells revealed a higher frequency of IFN-γ^+ RV-$CD4^+$ T cells in most healthy adults analyzed and of IFN-γ^+ RV-$CD8^+$ T cells in some of them (Fig. 5A), suggesting that $CD25^+$ Treg cells control the RV-T cells. This finding is in agreement with the fact that in RV infected mice $CD4^+$ $CD25^+$ cells suppress specific and non-specific B and T cells responses after the virus clearance (Kim et al., 2008). The absence of any increment in IFN-γ^+ RV-T from some healthy volunteers could be related to the removal of recently activated $CD25^+$ effector T cells or to a lack of recent RV stimulation. The $CD25^+$ and $CD25^-$ Treg cells could be mediating their function through mechanisms that involve membrane bound or soluble TGF-β (Nakamura et al., 2004), soluble IL-10 (Sun et al., 2007; Vieira and O'Garra, 2007) or other molecules (Vignali et al., 2008). In our model, PBMC from most volunteers depleted of $CD25^+$ cells and also treated simultaneously with ALK5i had higher frequencies of RV-$CD4^+$ and $CD8^+$ T cells than PBMC with $CD25^+$ cells depletion or ALK5i treatment alone (Fig. 5G–H), suggesting that the TGF-β is derived from cells other than $CD25^+$ Treg cells. As previously suggested, the variability in the T cell response among healthy volunteers could be related to differences in recent RV exposure. The $CD25^+$ independent source of TGF-β in our studies could potentially be $CD25^-$ Treg cells, and specially monocytes, most of which express membrane bound LAP (data not shown).

Contrary to adults, TGF-β is not involved in modulating RV-T cells in children (Fig. 6C). However, $CD4^+CD25^+CD127^{low}LAP^+$ Treg cells were observed in peripheral blood of all children studied (Fig. 7E–F) suggesting that, although present, they are not involved in a TGF-β–mediated regulation of peripheral RV-T cells. To our knowledge, absolute values of Treg cells have not been previously reported in young (<2 year old) children and few studies have addressed relative frequencies of Treg cells subpopulations (Fuchizawa et al., 2007). However, the relative percentage of $CD4^+CD25^+$ and probably $CD4^+CD25^+CD127^{low}$ T cells (the latter is equivalent to FoxP3$^+$

cells) (Seddiki et al., 2006) we found in children with GE are similar to those reported for the same age group in healthy children, suggesting that GE, and RV GE in particular, do not change the frequencies of these peripheral Treg cells (Fuchizawa et al., 2007).

It is possible that although Treg cells are present, they cannot perform their function in our assay in children. In support of this hypothesis, depletion of $CD25^+$ Treg cells from 7- to 25-month-old HIV infected and exposed infants did not increase the frequency of HIV specific T cells (Legrand et al., 2006). In addition, in similar studies of *N. meningitidis* specific T cells, $CD25^+$ Treg cells are not apparent in 2- to 7-year-old children, but are detectable in children older than 8 years and at higher levels in adults (Davenport et al., 2007). These findings could be related to immaturity of Treg from children. In fact, some studies, but not others, have shown that, compared to circulating Treg cells from adults, cord blood Treg cells do not modulate effector T cell responses to mitogens (Fritzsching et al., 2006; Takahata et al., 2004), alloAg (Santner-Nanan et al., 2008; Thornton et al., 2004; Wing et al., 2005), auto-Ags and foreign Ags (Brustoski et al., 2006; Legrand et al., 2006; Wing et al., 2005). Cord blood Treg cells are phenotypically naive ($CD45RA^+$ $CD38^+$), while adult Treg cells have a memory phenotype ($CD45RO^+$ $HLA\text{-}DR^+$) (Takahata et al., 2004; Wing et al., 2002) and when naive $CD45RA^+$ Treg cells from cord blood and peripheral blood from adults (Fritzsching et al., 2006) are compared, they showed similar reduced regulatory responses compared to $CD45RO^+$ adult Treg cells (Fujimaki et al., 2008). Taking into account that most of the Treg cells in our study children have a naive phenotype (Fig. S3), this could explain why the ALK5i treatment did not reveal a higher frequency of IFN-γ^+ $CD4^+$ T cells in response to RV (Fig. 5C). It is important to note that the regulatory function of cord blood Treg cells becomes more apparent in assays in which effector responses are evaluated to strong stimulus such as polyclonal ones (Chang et al., 2005; Thornton et al., 2004); this observation could explain the increments in the frequencies of IFN-γ^+ $CD4^+$ T cells in response to SEB in some children (Fig S2).

Lack of detection of RV-T cells in children is intriguing, because 75% of them have antigenemia (Table S1) and for this reason, one would expect at least a systemic T cell response. Of note, the few children from group C with IFN-γ^+ RV-$CD4^+$ T cells (Fig. 3) response are equally distributed among children with and without antigenemia (data not shown). Other studies are necessary to clarify why RV specific T cells are so low in children with RV GE; and especially, to explore the local immune response to RV in the human GALT.

In conclusion, our results show that in healthy adults RV-$CD4^+$ T cells predominantly produce IFN-γ^+ and not IL-2, and that IFN-γ^+ $CD4^+$ and to a lesser extent $CD8^+$ T cells are modulated by $CD25^+$ Treg cells and by a TGF-β-dependent regulatory mechanism (independent from $CD25^+$ Treg cells). In contrast, children with acute RV GE have few or no circulating RV-T cells and regulation by TGF-β cannot explain this finding.

Materials and methods

Subjects and specimen collection

Three groups of individuals were enrolled in this study: healthy adult volunteers (H) ($n=21$; 11 female and 10 male; median age = 26 years, range: 20–49), adults with acute RV GE ($n=5$; 4 female and 1 male; median age = 22 years; range: 21–34) and children with diarrhea ($n=53$; 14 females and 39 males; median age = 14 months; range: 4–22). Healthy adult volunteers and adults with RV GE were students from the Medical School and hospital or laboratory employees; adults with GE were studied during the acute (A) (1–7 days after onset of symptoms) and convalescence (C) (12–35 days after onset of symptoms) phases of the disease. Children with diarrhea were hospitalized at the moment of sample collection in the San Ignacio Hospital. All adult volunteers and parents or guardians of children signed an informed consent approved by the Ethics Committee of the Medical School of the Pontificia Universidad Javeriana. Peripheral blood samples were drawn with heparin and EDTA anticoagulants. Stool samples were collected from patients with diarrhea and stored at −70 °C. Blood samples with EDTA were used to obtain complete blood counts while samples with heparin were centrifuged at 400×*g* for 10 min to obtain plasma and cells. Plasma samples were stored at −70 °C and cells were diluted 1:3 with phosphate buffer solution (PBS; pH = 7.4; Cellgro, Lawrence, KS) to isolate PBMC using Lymphosep medium (*d = 1.077;* ICN Biomedical Inc. Irvine, CA). PBMC were washed twice with AIM-V media, (Gibco, Grand Island, NY) and used fresh in all experiments.

Detection of RV antigen, RV RNA and RV specific IgA

The presence of RV antigen in feces was determined using an ELISA previously described (Jaimes et al., 2002). A supernatant of the RF (P6[1]G6) strain of bovine RV-infected MA104 cells was used as a positive control and a known RV negative human fecal specimen was used as a negative control in each plate. Results were expressed as percentage of reactivity referred to the positive control. From all stool samples nucleic acids were extracted and RV P genotyping was performed as previously described (Mesa et al., 2007; Rojas et al., 2007). Plasma RV-specific IgA was detected by ELISA as previously described (Rojas et al., 2003) defining the titer of plasma IgA as the reciprocal of the last dilution exceeding an optical density of 0.1 that was twice the optical density of mock-treated wells. For detection of RV antigen in plasma, the same protocol described for feces was followed, with minor modifications: plasma samples were diluted in Blotto 2.5% and non diluted PBMC lysates were used (Crawford et al., 2006), and three instead of five washes were made; the RV antigen level was expressed as percentage of reactivity referred to the positive control.

Viruses and cells

The RRV strain (P5B[3] G3) of simian RV (obtained from Dr. H.B. Greenberg, Stanford University, CA) was grown in MA104 cells as previously described (Mesa et al., 2007). When RRV was used as a clarified MA104 cells culture supernatant (titre of 3.5×10^7 focus forming units [ffu]/ml) the supernatant of mock-infected MA104 cells was used as a control. RRV purified on a CsCl gradient was obtained as previously described (Mesa et al., 2007) and had titres between 3 and 8×10^7 ffu/ml. Before use, purified virus was dialyzed against RPMI (Gibco-BRL, Gaithersburg, MD) and the dialyzing media was kept to be used as control. For some experiments, the Wa strain (P1A[8] G1) of human RV (obtained from Dr. E. Mendez, UNAM, Mexico) was treated with 3 μg/ml trypsin (Sigma, St. Louis, MO) for 45 min at 37 °C and then grown in MA104 for 16 h at 37 °C. Cells were lysed by repeated freezing and thawing and then concentrated on a 40% sucrose cushion. The Wa stocks had titres between 2 and 3×10^8 ffu/ml. Supernatants of MA104 cells cultured without Wa and treated similarly were used as mock controls. The RNA patterns of both RV strains were confirmed by polyacrylamide gel electrophoresis.

Quantification of leukocytes and of Treg subpopulations

Absolute counts of total and subsets of leukocytes were determined using the Coulter hematology analyzers STKS or LH750. Two protocols were used for quantification and phenotyping of Treg cells and all monoclonal antibodies (MAbs) were from BD Biosciences (San Diego, CA) except where indicated. In the first protocol (stain 1), PBMC were stained with biotinylated goat anti-human LAP (TGF-β1) polyclonal Ab (R&D Systems; Minneapolis, MN) or with control biotinylated goat IgG (R&D Systems) at 4 °C for 30 min. After washing with staining buffer (0.5% bovine serum albumin [Merck, KGaA,

Darmstadt, Germany], 0.02% sodium azide [Mallinckrodt Chemicals, Paris, KY] in PBS) cells were stained with anti-CD4 FITC, anti-CD25 APC, anti-CD127 PE MAbs and PerCP conjugated Streptavidin for 30 min at 4 °C. Cells were washed and resuspended in 1% paraformaldehyde in PBS. Cells were acquired with a FACSCalibur flow cytometer (BD Biosciences) equipped with blue and red lasers and analysed with CellQuest Pro software. Dead cells and debris were excluded by forward and side scatter gating. Results are expressed as relative and absolute counts of $CD4^+CD25^+$, $CD4^+CD25^+CD127^{low}$, and $CD4^+CD25^+CD127^{low}LAP^+$ T cells (Fig. S3A). For the second protocol (stain 2), PBMC were washed with PBS and resuspended in 400 μl Aqua viability reagent (25 μg/ml Aqua [Invitrogen Molecular Probes, Eugene, OR] in PBS) for 30 min at 4 °C. Then cells were washed, FCγRs were blocked with 5 μl FCγR blocking reagent (Miltenyi Biotec Inc, Auburn CA) for 15 min at room temperature, and then stained with anti-CD3 Pacific Blue, anti-CD4 PerCPCy5.5, anti-CD25 APC, anti-CD45RA PECy7, anti-CD127 FITC (eBioscience, San Diego CA) and anti-LAP PE (R&D systems) or IgG1 isotype control-PE MAb (R&D Systems). Cells were incubated for 45 min at 4 °C, washed with staining buffer, acquired in a FACSAria flow cytometer (BD Biosciences) equipped with blue, red and violet lasers and analysed with FlowJo v.7.4 software. Results are expressed as relative and absolute counts of viable $CD3^+CD4^+CD25^+$, $CD3^+CD4^+CD25^+CD127^{low,}$ and $CD3^+CD4^+CD25^+CD127^{low}LAP^+$ T (Fig. S3B).

Depletion of $CD25^+$ cells

$CD25^+$-depleted PBMC were obtained using MACS CD25 MicroBeads (Miltenyi Biotec Inc) following the protocol previously described (Aandahl et al., 2004). To verify that $CD25^+$ cells were correctly removed, fractions of cells were stained before and after the depletion process. The fractions of $CD4^+CD25^{int}$ and $CD4^+CD25^{hi}$ cells removed were 61% or higher and 100%, respectively.

Inhibition of TGF-β activity

To block the activity of TGF-β, previously tittered 12 μg/ml recombinant human latent associated peptide (rhLAP) (R&D Systems) or 10 μM SB431542 (TGF-βRI/ALK5 inhibitor; ALK5i; Sigma or Tocris, Ellisville, MO) were added to the PBMC for 10 min and 30 min, respectively, at 37 °C before antigen stimulation.

Culture of PBMC and intracellular cytokine staining (ICS)

To evaluate the frequency of cytokine producing T cells, ICS was performed, as previously described (Jaimes et al., 2002). In this short term assay, only memory T cells have the capacity to respond and thus are the only cells detected. Briefly, 1×10^6/ml total PBMC were incubated with RV (RRV or Wa) (MOI = 7), control supernatant (mock) or 1.25 μg/ml superantigen, Staphylococcal enterotoxin B (SEB) (Sigma), in the presence of 0.5 μg/ml anti-CD49d MAb (BD Pharmingen) and 0.5 μg/ml anti-CD28 MAb (BD Pharmingen) for 10 h at 37 °C. For the last 5 h, 5.0 μg/ml Brefeldin A (Sigma) was added. The human RV strain, Wa (MOI = 7), was used in selected experiments to confirm the results with RRV. Cells were washed with PBS and 0.05 mM EDTA in PBS, fixed with 1% paraformaldehyde in PBS and then treated with FACS Permeabilizing Solution 2 (BD Biosciences). Then cells were stained for 30 min at 4 °C with FITC, PE, PECy7, PerCP, PerCP-Cy5.5, APC, APC-Cy7 or Pacific Blue-conjugated mouse MAbs to CD3, CD4, CD8, CD69, IFN-γ, IL-13, IL-10, IL-2 and IL-17. Cells were washed and resuspended in 1% paraformaldehyde in PBS, acquired on FACSCalibur or FACSAria Flow Cytometers (BD Biosciences) and analyzed with Cell Quest Pro, DIVA v.6.1 or FlowJo v.7.4 softwares. Results are reported as net percentage of cytokine positive, $CD69^+CD4^+$ or $CD69^+CD8^+$ cells after subtracting the values observed with cells stimulated with control antigens, which showed values with a median of 0.017%. Net values greater than 0.02% were considered positive for all cytokine producing T cells, according to the values observed in children without evidence of exposure to RV (group A children, see below). Due to limited amount of cells recovered from children and some adults, not all experiments were performed with cells from each individual. For this reason numbers of subjects vary between the different tables and figures.

Statistical analysis

Statistical analyses were performed with GraphPad Prism software v.5.0, using non-parametric tests. Differences between non-paired and paired results were compared with Mann–Whitney *U* tests and Wilcoxon tests, respectively. Kruskall–Wallis was used to determine differences among several groups followed by Mann–Whitney test to compare two groups. Chi square and Fisher exact tests were used for comparison of frequencies. Significance was established if $P<0.05$. Data are shown as median and range.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.12.016.

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